Effect of the season on the free phytoprostane content in Cornicabra extra virgin olive oil from deficit-irrigated olive trees

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Abstract

BACKGROUND: The effect of regulated deficit irrigation (RDI) on the phytoprostane (PhytoP) content in extra virgin olive (*Olea europaea* L., cv. Cornicabra) oil (EVOO) was studied. During the 2012 and 2013 seasons, T0 plants were irrigated at 100% ETc, while T1 and T2 plants were irrigated avoiding water deficit during phases I and III of fruit growth and saving water during the non-critical phenological period of pit hardening (phase II), developing a more severe water deficit in T2 plants. In 2013, a fourth treatment (T3) was also performed, which was similar to T2 except that water saving was from the beginning of phase II to 15 days after the end of phase II.

RESULTS: 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, 9-D_{1t}-PhytoP, 16-B_1-PhytoP and 9-L_1-PhytoP were present in Cornicabra EVOO, and their contents increased in the EVOO from RDI plants.

CONCLUSION: Deficit irrigation during pit hardening or for a further period of 2 weeks thereafter to increase irrigation water saving is clearly critical for EVOO composition because of the enhancement of free PhytoPs, which have potential beneficial effects on human health. The response of individual free PhytoPs to changes in plant water status was not as perceptible as expected, preventing their use as biomarkers of water stress.

INTRODUCTION

Olive trees are widely cultivated in Mediterranean coast countries, and olive oil consumption has increased from 1.67×10^6 t in 1990 to 3.09×10^6 t in 2011.[1] This strong increase could be due to the direct relationship between olive oil consumption and health benefits.[2,3]

According to the European regulation,[4] virgin olive oil means oil obtained solely by mechanical or other physical means under conditions that do not lead to alterations in the oil. In addition, extra virgin olive oil (EVOO) means virgin olive oil having a maximum free acidity, in terms of oleic acid, of 0.8 g per 100 g, and other characteristics detailed in the commission implementing regulation,[5] which contribute to the fact that these oils exhibit the best organoleptic features, with tastes and aromas which perfectly reproduce the fruit of origin. Moreover, EVOOs can have different proportions of oleic acid, polyphenols, squalene, triterpenes, tocopherols and other minor constituents, depending on cultivar,[2,6] season[2] and agricultural management, mainly irrigation,[6,7] fertilization[8,9] and harvesting date.[2,10]

Phytoprostanes (PhytoPs) are components of an oxidative injury-sensing, archaic signaling system which could not only be potentially used as markers of oxidative degradation of plant-derived foodstuffs but are also considered biologically

active molecules.[11,12] Although there is little information about the biological effects of PhytoPs, it is known that free PhytoPs are the only prostanes able to be absorbed by humans.[13] They have been identified in urine and found esterified to lipids in the plasma of healthy men consuming vegetable oil,[14] with some evidence that they can modulate the function of the immune and vascular systems.[15,16] Despite the fact that most olive orchards in the Mediterranean area have been traditionally grown under rain-fed conditions, it has been demonstrated that irrigation is a vital practice in improving both olive production and productivity.[17] Nevertheless, the Mediterranean coast is characterized by the aridity of the climate and the persistent shortage of water resources. In recent years, strong competition for the water that is available has arisen with other non-agricultural users. To cope with this water scarcity situation, deficit irrigation strategies such as regulated deficit irrigation (RDI) may offer an approach for saving water in woody crops by minimizing or eliminating negative impacts on yield and crop revenue.[18]

RDI strategies are accomplished by imposing water deficits during the phenological stages relatively tolerant to water stress (non-critical periods). Studies on olive trees have reported that pit hardening is the most resistant (non-critical) phenological period to drought.[19] Traditionally, it has been assumed that oil accumulation starts towards the end of the olive pit-hardening period.[20] However, recently, Pérez-López *et al.*[21] showed that olive oil accumulation starts a few days after the onset of pit sclerification rather after the end of massive pit hardening. Thus, at the end of olive pit hardening, the olive fruit has accumulated between 25 and 40% of its final oil content.

For the above reasons, this work was focused on (1) studying the effect of different water deficit levels during the early stages of oil accumulation in olive fruit (pit-hardening period) on free PhytoP levels, and whether a longer water deficit situation during olive oil accumulation (just after pit hardening) is able to enhance free PhytoP accumulation, and (2) evaluating the effect of season on the profile and contents of free PhytoPs in Cornicabra EVOO.

MATERIALS AND METHODS

Plant material and experimental conditions

The experiment was carried out in 2012 and 2013 on a farm near the city of Ciudad Real, Spain (39° 00′ N, 3° 56′ W, altitude 640 m). The plant material consisted of self-rooted 14-year-old olive trees (*Olea europaea* L., cv. Cornicabra). Tree spacing followed a 7 m × 4.76 m (300 trees ha^{-1}) pattern.

The soil is an alkaline (pH 8.1) and shallow Alfisol Xeralf Tipic Haploxeralf with a clay loam texture, low electrical conductivity (0.2 dS m^{-1}), organic matter (10.5 g kg^{-1}), nitrogen (1.2 g kg^{-1}) and potassium (17×10^{-4} mol kg^{-1}) and high cationic exchange capacity (0.186 mol kg^{-1}). A discontinuous petrocalcic horizon is located between 0.75 and 0.85 m. The soil water content for the first 0.3 m depth is 228 g kg^{-1} at field capacity (soil matrix potential −0.03 MPa) and 121 g kg^{-1} at permanent wilting point (soil matrix potential −1.5 MPa), whereas from 0.3 to 0.75 m it was 430 and 211 g kg^{-1} respectively. The orchard was managed under no-tillage conditions; weeds were controlled with post-emergence herbicides. Pest control and fertilization practices were those usually used by local growers. Irrigation was performed daily and during the night using a drip irrigation system with four emitters (each delivering 8 L h^{-1}) per tree and irrigation water with an electrical conductivity of 2.6–2.9 dS cm^{-1}.

Treatments

From mid-May to the end of September, control plants (T0 treatment) were irrigated at 100% ETc of the previous week. When midday stem water potential (Ψ_{stem}) values of that week were below −1.2 MPa before pit hardening (phase I of fruit growth) or −1.4 MPa during and after pit hardening (phases II and III of fruit growth respectively), irrigation amounts were increased by 10% in order to ensure no irrigation-related stress.[22] In addition to T0, two RDI treatments (T1 and T2) were applied, which were based on avoiding water deficit during phases I and III of fruit growth, maintaining Ψ_{stem} values around the threshold values indicated for T0 plants, and saving irrigation water during the non-critical phenological period of pit hardening (phase II), developing different situations of water deficit (threshold Ψ_{stem} values of −2.00 and −3.00 MPa in T1 and T2 plants respectively). In 2013, a fourth treatment (T3) was also performed, which was based in an irrigation protocol similar to that used for T2, except that the Ψ_{stem} threshold value of −3.00 MPa was used from the beginning of phase II to 15 days after the end of phase II.

The irrigation protocol in T1, T2 and T3 plants was based on that proposed by Moriana *et al.*[22] Irrigation amounts were determined weekly based on the Ψ_{stem} measurements and irrigation was begun when measured Ψ_{stem} values were lower than the threshold values suggested.

During the 2012 and 2013 irrigation seasons (days of year (DOY) 134–273 and 148–273 respectively), ETc was 202 and 183 mm respectively. Cumulative amounts of applied water in T0, T1 and T2 respectively, measured by means of flow meters integrated in the irrigation system, were 407, 196 and 141 mm in the 2012 season and 338, 164 and 112 mm in the 2013 season, whereas the irrigation water amount in T3 (2013) was 88 mm.

Extra virgin olive oil extraction

Olive fruits were harvested in mid-December (DOY 345 in 2012 and DOY 346 in 2013). EVOO was extracted using an Abencor system and the oil obtained was separated by decanting. Oil samples were filtered and stored in amber glass bottles without headspace at −18 °C in darkness until analysis.

Measurements

Climate, plant water status and yield

Meteorological data, namely air temperature, solar radiation, air relative humidity, rainfall and wind speed 2 m above the soil surface, were collected in a nearby automatic weather station. Mean daily air vapour pressure deficit (VPD_m) was calculated according to Allen *et al.*[23] Crop irrigation requirements (ETc) were estimated according to daily crop reference evapotranspiration (ETo), calculated using the Penman–Monteith equation,[23] and a crop factor based on the time of year[24] and taking into consideration canopy size.[25]

Midday (12:00 solar time) stem water potential (Ψ_{stem}) was measured on the middle third of the trees, in fully developed leaves from two trees of each replicate, enclosing leaves in small black plastic bags covered with aluminium foil for at least 2 h before measurements in the pressure chamber (Model 3005, Soil Moisture Equipment Co., Santa Barbara, CA, USA). Minimum daily leaf conductance values were measured with a steady state porometer (LICOR 1600, Lincoln, NE, USA) at midday on the abaxial surface of the leaves and in a similar number and type of leaves as used for the Ψ_{stem} measurements.

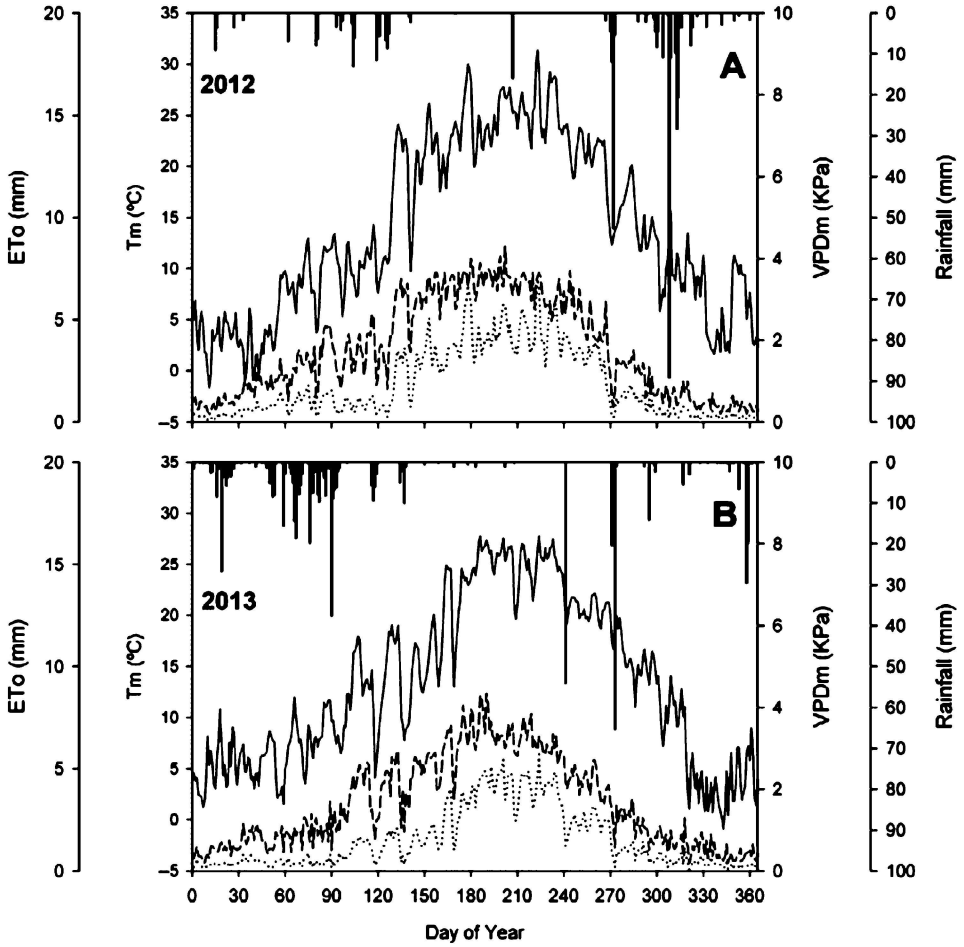

Figure 1. Daily mean temperature (Tm, –), crop reference evapotranspiration (ETo, – –), mean daily air vapour pressure deficit (VPDm, · · · · ·) and daily rainfall (vertical lines) during years (A) 2012 and (B) 2013.

In order to describe the cumulative effect of the water deficit, the water stress integral ($S_{\psi stem}$) was calculated from the Ψ_{stem} data using the expression proposed by Myers.[26] Moriana *et al.*[22] suggested the use of stem potential threshold values of −1.2 MPa before the beginning of pit hardening and −1.4 MPa from that moment to harvesting. This change improves the capacity of comparison between different experiments and/or locations. The expression used was

$$S_{\psi_{stem}} = |\sum (\overline{\psi}_{stem} - b) \times n|$$

where $\overline{\psi}_{stem}$ is the average Ψ_{stem} value for any interval, *b* is the stem potential threshold value and *n* is the number of the days in the interval.

Marketable olive fruits were harvested and the mean fruit weight was determined according to the weight and number of fruits per box in randomly selected boxes per replicate.

Chemicals and reagents

PhytoPs (9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, *ent*-16-F_{1t}-PhytoP, *ent*-16-*epi*-16-F_{1t}-PhytoP, 9-D_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, 16-B_1-PhytoP and 9-L_1-PhytoP, named according to the Taber/Roberts nomenclature, which was approved by IUPAC for identification of structures unequivocally[27,28]) were synthesized according to the published procedures.[27,29,30] Solid phase extraction (SPE) cartridges (Strata X-AW, 100 mg per 3 mL) were acquired from Phenomenex (Torrance, CA, USA). Bis-Tris (bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane) was obtained from Sigma-Aldrich (St Louis, MO, USA) and hexane from Panreac (Castellar del Vallés, Barcelona, Spain). Liquid chromatography/mass spectrometry (LC/MS)-grade solvents methanol and acetonitrile were purchased from J.T. Baker (Phillipsburg, NJ, USA).

Free phytoprostane extraction and UHPLC/QqQ-MS/MS analyses

EVOO samples (1 mL) were subjected to dilution and SPE using Strata X-AW cartridges, following the procedure described by Collado-González *et al.*[31] Briefly, samples diluted in 10 mL of *n*-hexane and rediluted in 2 mL of methanol were further diluted in 2 mL of Bis-Tris buffer and applied to previously conditioned and equilibrated cartridges, which were subsequently washed to remove unwanted compounds. Target compounds were eluted with methanol and dried using a SpeedVac concentrator (Savant SPD121P, Thermo Scientific, Waltham, MA, USA). Dry extracts were reconstituted with 200 µL of a mixture of A/B solvents (90:10, v/v), sonicated and filtered through a 0.45 µm filter (Millipore, Bedford, MA, USA). Samples (20 µL) were analysed in a UHPLC/QqQ-MS/MS system (Agilent Technologies, Waldbronn, Germany).

Free PhytoP separations were performed using a BEH C18 column (2.1 mm × 50 mm, 1.7 µm; Waters, Milford, MA, USA). The mobile phase used was a mixture of (A) water/acetic acid

(99.99:0.01, v/v) and (B) methanol/acetic acid (99.99:0.01, v/v). The flow rate (0.2 mL min^{-1} using a linear gradient), electrospray ionization (ESI) conditions and ion optics were as described previously.[31] The MS analysis was applied in multiple reaction monitoring (MRM) negative ESI mode. Data acquisition and processing were performed using MassHunter Version B.04.00 software (Agilent Technologies). For quantification of free PhytoPs, all PhytoPs synthesized were used.

Statistical design and analysis

The design of the experiments was completely randomized with four replications, each replication consisting of five adjacent tree rows, each with nine trees. Measurements were taken on the inner trees of the central row of each replicate, which were very similar in appearance (leaf area, trunk cross-sectional area, height, ground shaded area, etc.). Data were analysed using SPSS software.[32] Analysis of variance was performed and mean values were compared by Tukey's test ($P < 0.05$). Values for each replicate were averaged before the mean and standard error (SE) of each treatment were calculated. To check the regression model hypothesis (linearity, homoscedasticity, normality and independency), the Kolmogorov–Smirnov test was used with the Liliefors correction and the Shapiro–Wilk test for normality and the Levene test for homoscedasticity on the typified residuals. To do the Levene test, data were divided into two groups according to the median of abscissa data.

RESULTS

Climate, plant water status and fruit yield

During the irrigation season, average daily maximum and minimum air temperatures were 31.4 and 13.1 °C in 2012 and 31.3 and 12.7 °C in 2013 (Fig. 1). VPD_m ranged from 0.09 to 3.50 kPa in 2012 and from 0.09 to 2.85 kPa in 2013 (Fig. 1). Accumulated ETo was 856 and 721 mm in 2012 and 2013 respectively (Fig. 1). There were important differences in rainfall in 2012 and 2013, with levels of 90 and 326 mm respectively before the beginning of the irrigation seasons and 96 and 159 mm respectively during the irrigation season (Fig. 1).

In both seasons, $S_{\psi stem}$ values in all irrigation treatment plants increased progressively during the measurement period, with the characteristic that the highest increases were observed mainly in T1, T2 and T3 plants during pit hardening, coinciding with deficit irrigation (Fig. 2). $S_{\psi stem}$ values in T0, T1 and T2 were higher in 2012 than in 2013 (Fig. 2). In 2012, $S_{\psi stem}$ values in T1 and T2 plants were similar and, from the final stage of pit hardening to the end of the measurement period, higher than those in T0. In 2013, from the beginning of pit hardening, significant differences between treatments were found, with T3 plants showing the highest and T0 plants the lowest $S_{\psi stem}$ values, while T1 and T2 plants showed $S_{\psi stem}$ values similar to those in T0 and T3 plants (Fig. 2).

No differences between treatments were observed in leaf conductance values except during the pit-hardening period, when on some dates significant differences between treatments were observed (Fig. 3). On those dates, T0 plants reached leaf conductance values higher than those of RDI (T1, T2 and T3) plants, which showed similar leaf conductance values (Fig. 3).

No differences between treatments were found in fruit yield, mean crop load and average fruit weight in both seasons (Table 1). However, significant differences between seasons were found in fruit yield and mean crop load because of the very low yield in 2012 (Table 1).

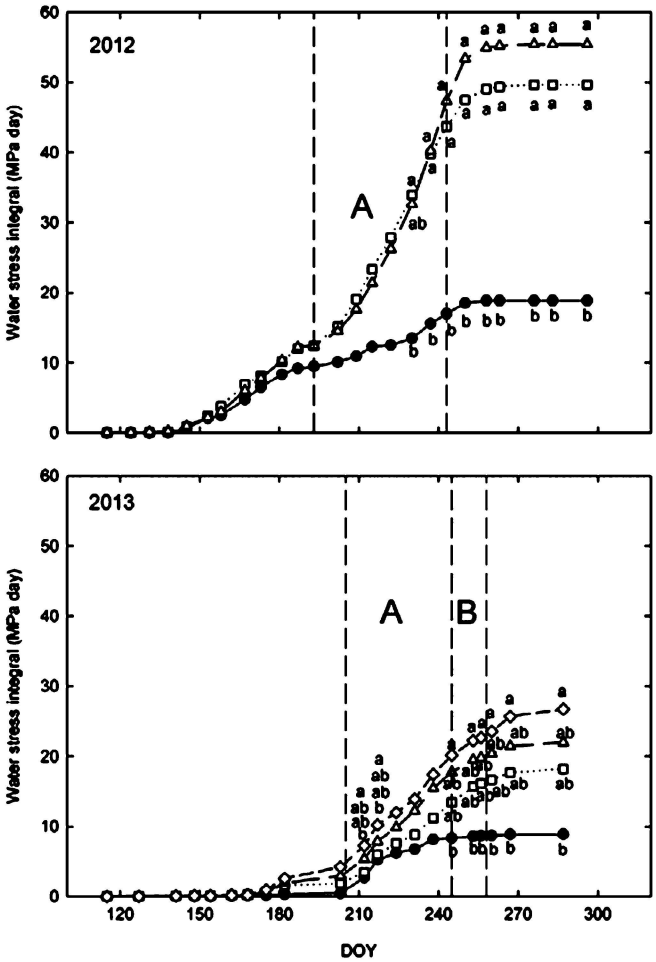

Figure 2. Water stress integral in T0 (●, –), T1 (□, ····), T2 (△, – –) and T3 (◇, - - -) during 2012 and 2013 irrigation seasons. Different letters on data points at each date indicate significant differences according to Tukey's test ($P \leq 0.05$). Broken vertical lines indicate pit hardening (A) and 15 days after pit hardening (B).

Free phytoprostane profile

Identification of free PhytoPs was performed on the basis of their pseudomolecular ion (*m/z* 327.2, 325.2 and 307.2) and their elution order according to their retention times as described in Fig. 4. The PhytoP profile of the Cornicabra EVOO is shown in Fig. 4, which indicates the presence of 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, 9-D_{1t}-PhytoP, 16-B_1-PhytoP and 9-L_1-PhytoP.

Water deficit and season effects on free PhytoP content

The total content of free PhytoPs in the Cornicabra EVOO from the different irrigation treatments ranged from 9.18 to 19.31 ng mL^{-1} in 2012 and from 31.92 to 67.87 ng mL^{-1} in 2013, with F_{1t}-PhytoP being the dominant PhytoP class and the D_1-PhytoP class the minor component (Table 2). The total free PhytoP content in the oil increased as a result of the water deficit effect, although no significant differences between T1 and T2 contents in 2012 and T1, T2 and T3 contents in 2013 were found (Table 2). Also, the results indicated that the highest total free PhytoP contents in olive oil from the different irrigation treatments were obtained in the season of maximum yield (Tables 1 and 2).

The response of each free PhytoP to water stress was not the same and some inter-seasonal differences were observed (Table 2).

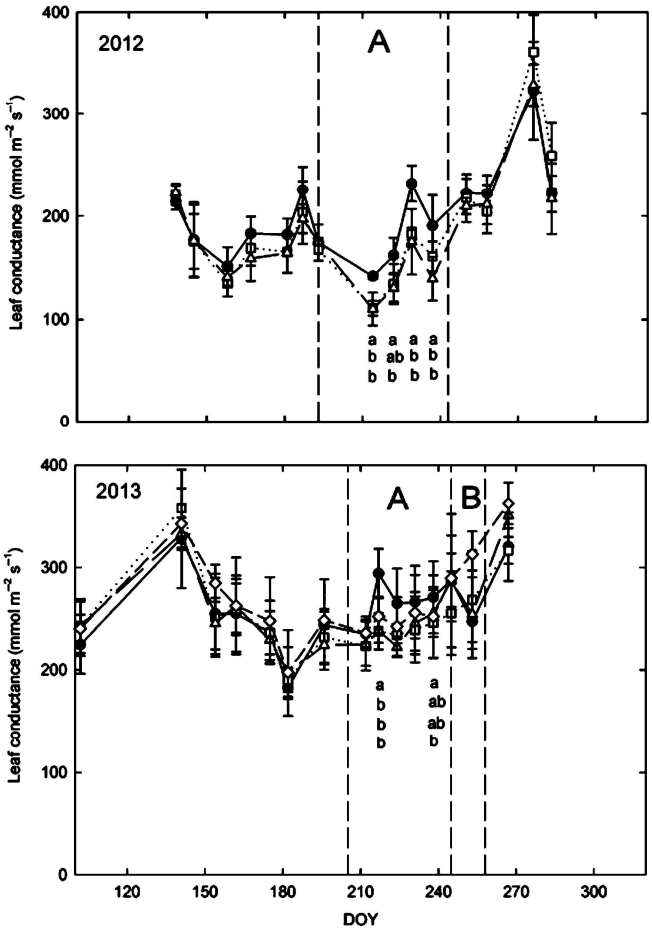

Figure 3. Midday leaf conductance values (mean ± SE, not shown when smaller than symbols) in T0, T1, T2 and T3 during 2012 and 2013 irrigation seasons. Symbols as in Fig. 2.

For example, 9-*epi*-9-F_{1t}-PhytoP content showed a similar response to water deficit to that observed in total free PhytoP content in both seasons, 9-F_{1t}-PhytoP showed similar content in T1 and T2 in 2012, whereas a gradual increase from T0 to T2 was observed in 2013, and 9-D_{1t}-PhytoP showed different content in T1 and T2 in 2012 and a content in T1 similar to that observed in T0 and T2 in 2013 (Table 2).

Similarly, the inter-seasonal behaviour of some free PhytoPs was different from that observed for total free PhytoPs (Table 2). In this sense, the dominant free PhytoPs (9-F_{1t}-PhytoP and 9-*epi*-9-F_{1t}-PhytoP) showed a behaviour similar to that observed for total free PhytoP content. In contrast, 9-*epi*-9-D_{1t}-PhytoP and 16-B_1-PhytoP contents in the different irrigation treatments were similar in both seasons. Furthermore, 9-L_1-PhytyoP content in T1 and T2 and 9-D_{1t}-PhytoP content in T2 were higher in 2012 (Table 2).

Results showed that total and individual contents of free PhytoPs in EVOO from all irrigation treatments applied in 2012 and 2013 presented significant first-order correlations with respect to achieved $S_{\psi stem}$ values, except the correlations obtained in 2012 for 9-F_{1t}-PhytoP and 9-D_{1t}-PhytoP (Table 3). These equations were characterized by (1) very low values of slope in most of them, except in the equations of the dominant free PhytoP class (F_{1t}-PhytoP) in 2013, which showed the highest slope values, (2) the highest significant correlations in 2012 and 2013 being found

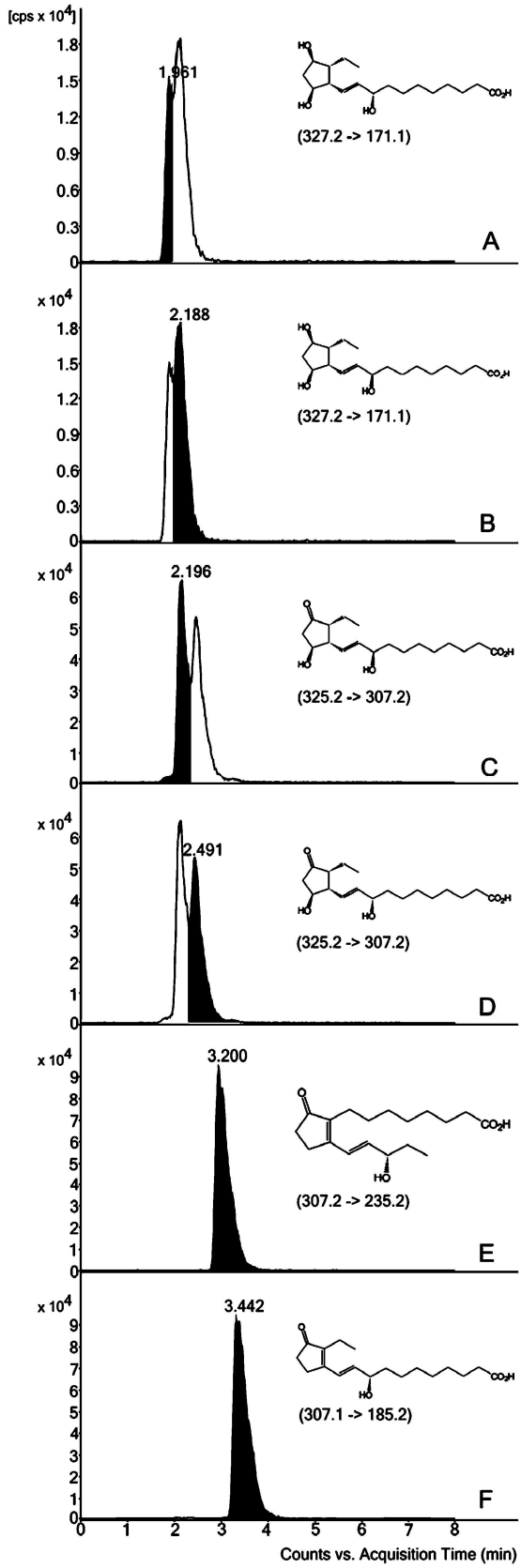

Figure 4. UPHLC/QqQ-MS/MS chromatograms of PhytoPs detected in olive oil, namely 9-F_{1t}-PhytoP (A), 9-*epi*-9-F_{1t}-PhytoP (B), 9-*epi*-9-D_{1t}-PhytoP (C), 9-D_{1t}-PhytoP (D), 16-B_1-PhytoP (E) and 9-L_1-PhytoP (F), and their preferential MRM transitions.

Table 1. Effect of irrigation treatments (T0, T1, T2 and T3) on Cornicabra olive fruit yield, mean crop load and average fruit weight in 2012 and 2013 seasons

Season	Treatment	Yield (kg per tree)	Crop load (number of fruits per tree)	Fruit weight (g)
2012	T0	1.95aB	780aB	2.50aA
	T1	4.88aB	2228aB	2.19aA
	T2	3.02aB	1135aB	2.66aA
2013	T0	31.92aA	16583aA	1.93aA
	T1	28.74aA	14468aA	1.99aA
	T2	31.01aA	16204aA	1.91aA
	T3	31.02a	16684a	1.86a

Means within a column followed by different capital letters and within a column for each season followed by different lowercase letters are significantly different at $P = 0.05$ by Tukey's test.

for 16-B_1-PhytoP and 9-F_{1t}-PhytoP respectively and (3) all significant first-order equations predicting a positive free PhytoP value in the range of $S_{\Psi stem}$ studied (Table 3).

DISCUSSION

Taking into consideration ETc and irrigation water levels applied in the 2012 and 2013 seasons, T0 plants were irrigated above crop water requirements (202 and 185% ETc respectively). Moreover, deficit-irrigated plants reduced the seasonal water applied with respect to estimated ETc by 3 and 10% in T1 plants and 30 and 39% in T2 plants in 2012 and 2013 respectively and by 52 % in T3 plants in 2013.

To explain why $S_{\Psi stem}$ and leaf conductance values in all treatments in 2012 were significantly higher and lower respectively than those in 2013 in spite of the fact that irrigation water amounts were similar in the two seasons (Fig. 2), it is key to take into account the scarcity of rainfall in 2012 and that the drip irrigation system in the olive orchard was installed in March 2012. Thus in most of the 2012 season the root system dissemination could have been that typical of olive trees under rain-fed conditions, whereas in 2013 a very important proportion of the root system could be confined to the volume of soil wetted by drip irrigation.[33] Consequently, the irrigation efficiency in most of 2012 could be lower than that in 2013, leading to $S_{\Psi stem}$ and leaf conductance values in T0, T1 and T2 clearly higher and lower respectively in 2012 than in 2013 (Fig. 2). Additionally, the very low yield and crop load in 2012 (Table 1) could contribute to the fact that $S_{\Psi stem}$ and leaf conductance values were higher and lower respectively than those in 2013.[34,35] The fact that rainfall reached 485 mm during the 2013 irrigation season can explain why the differences in $S_{\Psi stem}$ and leaf conductance values between treatments were not as important as expected (Fig. 2).

The very important difference in the yield obtained in the two seasons (Table 1) matches with the better adaptation of the trees to the drip irrigation system in 2013 and with the characteristic alternate bearing pattern of olive trees, which exhibits a frequency and intensity regulated by the genotype and growing conditions.[36] The increase in 2013 fruit yield seemed to be due to the higher number of fruits, because the average fruit weight was similar in the two seasons (Table 1). The fact that in both seasons no effect of T1 and T2 on fruit yield was observed confirms that the pit-hardening phenological period is not a critical period from the yield point of view.[19] However, the increase in total free PhytoP content in Cornicabra EVOO due to T1 and T2 clearly indicated that the pit-hardening period is critical for the PhytoP content in olive oil. Moreover, the fact that the yield was not affected and the free PhytoP content in EVOO increased in response to T3 indicated that it is possible to extend the water deficit period by 2 weeks after pit hardening, leading to a similar effect to that of T1 and T2 but increasing irrigation water saving (Tables 1 and 2).

The increase in free PhytoP content in EVOO from deficit-irrigated trees (T1, T2 and T3) could be related to stomatal regulation (Fig. 3) and the concomitant limitation on CO_2 fixation under water stress, which enhances reactive oxygen species formation[37] and promotes the formation of various lipid peroxidation products, including PhytoPs.[38]

It is difficult to understand why the highest total free PhytoP content in the EVOO from the different irrigation treatments was obtained in the season of better plant water status (Fig. 2, Table 2). In this sense, Berenguer *et al.*[39] showed that the level of linolenic acid, the precursor of PhytoPs, increased significantly with irrigation in one year out of two. However, other authors have found inconsistent fluctuations in some fatty acids from year to year.[39–41] In any case, it can be considered that the composition of EVOO results from a very complex multivariate interaction between the genotypic potential and the environmental, agronomic and technological factors that characterize fruit growth and ripening as well as oil extraction and storage.[20,42] Thus the minor components in EVOO may vary independently, depending on factors that are not always interrelated.

Thoma *et al.*[38] and Loeffler *et al.*[12] indicated that free PhytoPs are excellent biomarkers of oxidative degradation of plant-derived foodstuffs. In this sense, total and individual free PhytoP contents in Cornicabra oil increased as a result of water stress (Table 2). However, this response was not as perceptible as expected, because most correlations in Table 3 present very low values of slope, indicating that a large change in $S_{\Psi stem}$ is needed to get any change in individual free PhytoP contents. Also, it is important to consider that most correlations for individual free PhytoPs changed from one season to another (Table 3). In the light of these results, it is difficult to conclude that these individual free PhytoPs can be used as biomarkers of water stress, probably owing to the fact that the only requirement for forming PhytoPs is the presence of linolenic acid and molecular oxygen. Nevertheless, considering that there is some evidence that PhytoPs are biologically active lipids,[11,16,43] it is clear that the overall significant increase in free PhytoPs constitutes a potential nutritionally beneficial aspect of Cornicabra EVOO from trees cultivated under water deficit conditions during summer.

CONCLUSION

The results showed for the first time that water deficit during pit hardening or for a further period of 2 weeks thereafter to increase irrigation water saving is clearly critical for EVOO composition because of the enhancement of free PhytoPs. Moreover, an important inter-seasonal change in PhytoP content was observed, probably due to a very complex multivariate interaction between factors that are not always interrelated. Both circumstances are crucial for potential beneficial effects of Cornicabra EVOO on human health. The response of individual free PhytoPs to changes in plant water status was not as perceptible as expected, preventing their use as biomarkers of water stress when it is evaluated using $S_{\Psi stem}$ values.

Table 2. Effect of irrigation treatments (T0, T1, T2 and T3) on PhytoP contents (ng mL^{-1}) in Cornicabra EVOO in 2012 and 2013 seasons

Phytoprostane	Year	Treatment			
		T0	T1	T2	T3
9-F_{1t}-PhytoP	2012	5.24bB	10.00aB	9.73aB	-
	2013	19.31cA	32.61bA	39.19aA	39.26a
9-*epi*-9-F_{1t}-PhytoP	2012	2.83bB	4.86aB	5.26aB	–
	2013	11.68bA	23.01aA	27.28aA	25.81a
9-*epi*-9-D_{1t}-PhytoP	2012	0.13bA	0.13bA	0.26aA	–
	2013	0.16bA	0.22aA	0.23aA	0.23a
9-D_{1t}-PhytoP	2012	ND	0.19bA	0.45aA	–
	2013	0.12b	0.15abA	0.21aB	0.22a
16-B_1-PhytoP	2012	0.32bA	0.72aA	0.75aA	–
	2013	0.41bA	0.52 abA	0.63aA	0.60a
9-L_1-PhytyoP	2012	0.66bA	1.28bA	2.88aA	–
	2013	0.25bA	0.25bB	0.34aB	0.34a
Total	2012	9.18bB	17.18aB	19.31aB	–
	2013	31.92bA	56.76aA	67.87aA	66.45a

Means within a row for each PhytoP and season followed by different lowercase letters and within a column for each PhytoP and treatment followed by different capital letters are significantly different at $P = 0.05$ by Tukey's test. ND, not detected.

Table 3. Intercept (*a*), slope (*b*), coefficient of determination (r^2), number of data points (*n*) and mean square error (MSE) of first-order linear equations ($y = a + bx$) between each PhytoP content (ng mL^{-1}) and water stress integral (MPa day) in phases II and II, using all data pooled

Phytoprostane	Year	*a*	*b*	r^2	*n*	MSE
9-F_{1t}-PhytoP	2012	5.4214^{*} (1.6274)	0.0965^{NS} (0.0477)	0.3694^{NS}	9	5.3702
	2013	17.1334^{***}(3.3534)	0.8919^{***}(0.1771)	0.6444^{***}	16	29.2136
9-*epi*-9-F_{1t}-PhytoP	2012	2.6388^{**}(0.6010)	0.0558^{*}(0.0176)	0.5895^{*}	9	0.7324
	2013	9.8121^{**}(2.7128)	0.6999^{***}(0.1432)	0.6304^{***}	16	19.1181
9-*epi*-9-D_{1t}-PhytoP	2012	0.0881^{NS} (0.0394)	0.0028^{*}(0.0012)	0.4535^{*}	9	0.0031
	2013	0.1597^{****} (0.2207)	0.0028^{*}(0.0012)	0.2960^{*}	16	0.00013
9-D_{1t}-PhytoP	2012	-0.075^{NS} (0.2529)	0.0098^{NS} (0.0061)	0.3868^{NS}	9	0.0202
	2013	0.1022^{***}(0.0235)	0.0042^{**} (0.0012)	0.4542^{**}	16	0.0014
16-B_1-PhytoP	2012	0.2439^{*}(0.0858)	0.0117^{**}(0.0025)	0.7571^{**}	9	0.0149
	2013	0.3609^{****} (0.0477)	0.0102^{**}(0.0025)	0.5391^{**}	16	0.0059
9-L_1-PhytyoP	2012	0.3320^{NS} (0.0609)	0.0424^{*}(0.0178)	0.4471^{*}	9	0.7511
	2013	0.2191^{****} (0.0293)	0.0042^{*}(0.0016)	0.3413^{*}	16	0.0022
Total	2012	8.6264^{*}(2.5854)	0.2194^{*}(0.0757)	0.5456^{*}	9	13.5541
	2013	27.7895^{***}(5.9164)	1.6130^{***}(0.3124)	0.6557^{***}	16	90.9377

Values are mean ± SE (in parentheses). Significance:
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; NS, non-significant.

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